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PRINCIPAL INVESTIGATOR:  Thomas M. Mack, M.D.

CONTRACTING ORGANIZATION:  University of California
                          Los Angeles, California   90033

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Thomas M. Mack, M.D.

| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Southern California
Los Angeles, California 90033 | 8. PERFORMING ORGANIZATION REPORT NUMBER |
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Archived tissue blocks are being sought on breast cancer concordant twin pairs (207 MZ and 131 DZ) and on discordant pairs (549 MZ), the presence of selected biomarkers (p53, HER-2/*neu*, ER, and PR) in the tumor tissue is being determined, and the association of environmental and genetic factors with the development of these markers is being investigated. Blocks from 276 twins (200 from concordant pairs and 76 from discordant pairs) have been received. Based on 40 pairs of the concordant twins with completed immunohistochemistry, intra-pair analyses have shown that ER was the biomarker most likely to be identical in the pair (Kappa=0.42) and HER-2/*neu* was the least likely (Kappa=0.19). The relatively low agreement between members of these pairs suggests that environmental factors are likely to play a role in the development of these tumor markers. Preliminary analyses of the association of breast cancer risk factors with the tumor markers indicated that cases who breast fed were less likely to develop a tumor which expressed p53 than those who did not. During the next year, collection of tissue blocks and immunohistochemistry will be supplemented by sequencing of the p53 gene and FISH to determine HER-2/neu gene amplification. Additional epidemiologic analyses will be completed to further investigate the relationship of risk factors to tumor marker development.

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# A. INTRODUCTION

Abnormalities relating to the p53 gene are the most commonly found genetic aberration in breast cancer tumors, and include overexpression of p53 protein, loss of heterozygosity at the p53 locus, and specific mutations in the p53 gene. However, it is unknown why do some tumors have these changes and others do not. Further, little is known about what factors are involved in the interaction of oncogenes such as HER-2/neu with p53.

While investigators in previous studies have attempted to link p53 abnormalities to tumor histology, survival time, estrogen and progesterone receptor status, Her-2/neu, and, in some cases, risk factors for breast cancer, none has studied all of these factors within a large population of twins. These subjects offer great potential for distinguishing the role of predisposing genetic factors from environmental exposures. Specifically we will address the following issues in this study: 1) Are genetically similar tumors more likely to occur among identical twins than among fraternal twins? 2) Do environmental factors predispose to concordance or discordance of genetic abnormalities? 3) Do fraternal twins, concordant for environmental exposures, tend to be discordant for genetic abnormalities, suggesting that other predisposing genetic factors that can be identified? 4) Among identical twins discordant for disease, are specific environmental factors more related to tumors with a genetic abnormality than those without?

Three methods have been commonly used to detect p53 abnormalities: immunohistochemical methods of detecting overexpression of the mutant p53 protein, polymerase chain reaction (PCR) techniques for the detection and sequencing of specific p53 mutations, and Southern blots to detect loss of heterozygosity (LOH) at the p53 gene locus. Studies have indicated that 50-60% of breast tumors may have LOH in the 17p region; there may be overexpression of the p53 mutant protein in 27-54% of all breast tumors (3). Specific mutations in the p53 gene usually occur in the highly conserved exons 5-8 (4,5). Twenty-five percent have been shown to occur in codons 245, 248, 273, and 282 (6). From collaborative efforts of specific p53 mutations in more that 30 types of cancer it has been shown that different types of cancer evince different patterns of DNA base substitutions (7).

Rarely have all types of abnormalities been investigated within the same tumor tissue, but a few studies provide information on the correlations between them. Overexpression of the mutant p53 protein product has been seen in association with mutation of the p53 gene (8) but not invariably (9). LOH and overexpression of the p53 protein have been found to occur independently (9,10,11). The mechanism by which dysfunction in the p53 gene leads to malignant transformation is therefore unclear.

Under one hypothesis it would be necessary for both copies of the p53 gene to be inactivated by loss or mutation to prevent the transcription of the normal or 'wild-type' protein and hence prevent normal function of the gene. The failure by some investigators to demonstrate damage to or loss of both copies of the p53 gene suggests that additional steps or other mechanisms must precede malignant transformation. For example, under a hypothesis of co-dominance, a stable

mutant protein might bind to and inactivate any wild-type protein produced (12). Strong immunohistochemical staining for p53 in normal cells has been found in a mother and daughter with a family history of breast cancer (13). However, no p53 overexpression was found in fibroblasts from individuals from families with the Li-Fraumeni syndrome who had germline DNA mutations of the p53 gene (14). Thus another event (apart from damage to p53) sometimes may be necessary for expression of mutant protein, or only certain mutations in p53 may be related to overexpression of the mutant protein and subsequent malignant transformation.

Another mechanism by which the normal function of p53 gene may be interrupted is by nuclear exclusion (15). When p53 protein is found in the nucleus of cells, mutations in the gene are usually found, whereas when the protein is found in the cytoplasm, mutations are generally not found. If the protein is sequestered in the cytoplasm (by binding with heat shock proteins) then it may be unable to regulate nuclear division. Some studies have shown p53 protein to occur in the cytoplasm of lobular breast cancers (16).

When p53 mutations in germline tissue were found in members of Li-Fraumeni families (17), efforts to detect germline mutations in other high-risk families were intensified, largely without success (18, 19, 20). While these studies were based on small numbers of families: 5 (18) and 25 (19), or cases: 19 individuals with bilateral disease (20). This failure has led to the presumption that environmental factors or other genes may also determine the abnormalities in the p53 gene that lead to breast cancer (21). In any event, the inactivation or disabling of the p53 gene appears to be an important step in a large proportion of breast cancer cases, and studies have shown it to be an early step, present in *situ* tumors and maintained throughout all stages of tumor progression (8).

Since the etiology of breast cancer appears to be complex and heterogenous, other genes, especially oncogenes, may sometimes interact with p53 in the development and progression of breast cancer. HER-2/neu (or also referred to as c-erbB-2), located on the long arm of chromosome 17 (17q12-21.32) has been shown to occur in 20% of invasive breast cancer tumors and in 50% of all ductal carcinoma in situ (22). Studies that have examined the association of p53 with HER-2/neu have produced mixed results; at least four have found the two to be correlated (23, 24, 25, 26), while others have not (27, 28). Barbareschi et al. (26) suggest that p53 and HER-2/neu alterations may occur independently and at an early stage of tumor progression. Escape from hormonal control may be associated with HER-2/neu overexpression (which has been related to estrogen receptor negative tumors); while alterations in p53 may induce a high proliferation rate, leading to tumor progression and further opportunities for genetic damage.

The association of p53 abnormalities and HER-2/neu overexpression with estrogen and progesterone receptor status, histology, progression, and patient survival may provide insights into the mechanisms of tumor development and progression. While some studies have linked p53 overexpression to tumors with a more aggressive phenotype (28), it may be that LOH is more critical to tumor progression than any specific mutation (11). Nuclear p53 expression has been associated with tumors of aggressive (ductal) as well as less aggressive (medullary) histology (16); however neither LOH nor specific mutation sequences were assessed. HER-2/neu is generally found in association with a poorer prognosis(29).

The relationship of p53 and HER-2/neu overexpression to environmental and other genetic risk factors has not been extensively studied. A higher proportion of tumors with p53 protein expression in familial than in sporadic cases has been reported (30). p53 has been associated with low levels of estrogen receptors (23, 26, 28) and late age at first full term pregnancy has been linked to the prevalence of estrogen receptors (McTiernan et al., 1986). An effect of breast-feeding on risk has been found to be dependent on expression of HER-2/neu (32).

To assess the interrelationships of tumor suppressor genes, oncogenes, specific mutations, loss of heterozygosity, and protein overexpression, it is essential that all factors be examined in the same material. This study presents the opportunity to study the several characteristics of breast cancer tumors in a large group of familial cases--concordant twin pairs--and relate these findings to genetic identity and to environmental risk factors. Secondly, a large number of disease discordant identical twin pairs offers the opportunity to further study association of environmental factors with specific genetic changes in breast cancer tumors.

## B. BODY

Work done during the third year of the project has included the following:

1) Data management

2) Contact with twins from the International Twin Registry to obtain consent and release forms for acquisition of tissue blocks: Ongoing efforts to obtain consent and release forms from concordant MZ twin pairs, concordant DZ pairs, and discordant DZ pairs.

3) Ongoing correspondence with hospitals to borrow tissue blocks and return them after slides have been made.

4) Laboratory procedures
   a. Logging in of received blocks and slides in database.
   b. Ongoing processing of tissue blocks to cut slides and storage of them
   c. p53, HER-2/neu, and estrogen/progesterone receptor immunohistochemistry.

5) Results

**Data Management**
A data management system using SAS was set up in the first year of the study and has been used to monitor the correspondence and follow-up efforts with the twins and their hospitals. The laboratory information and the epidemiologic questionnaire information obtained from the twins when they first became part of the Registry have been linked using SAS datasets.

**Contact with Twins**

Three groups of twins have been contacted and results are shown in Table 1. There are a total of 1,225 cases for whom we are seeking archived tissue blocks. Our procedures for contacting the twins are the same for each group. Beginning with those who were diagnosed after 1975 and for whom we had already obtained pathology reports, we sent a letter explaining the study, the informed consent, and a release form to each twin for her signature. If we determined that a twin was deceased, these forms were sent to her next of kin. If we did not receive a response from a twin after 4 weeks, we have called the twin to be sure they received the forms and to answer any questions. Additional follow-up has been performed as required. For those with diagnosis dates before 1975, we called the hospitals first to determine if the tissue blocks were still available, before initiating the correspondence with the twin. Of the 85 hospitals called, blocks were available for approximately 30%.

We have resolved 570 cases at this time with tissue blocks obtained from 276. The largest group pending is with the effort to obtain the consent and release form from the patient or next of kin. We have been making extensive follow-up efforts on this group and for those who cannot be located, and for those who are known to be deceased with no next of kin identified, we will be sending a request for the tissue blocks directly to the hospital.

1) **MZ concordant twins:** 207 pairs of identical female twins, concordant for breast cancer, were initially selected to obtain archived tissue blocks. From these 414 cases we have obtained tissue blocks from 164 or 39.6%. We have continued to follow-up our contacts with these twins and their next-of-kin to obtain consent and release forms.

2) **DZ concordant twins:** We initiated efforts to obtain consent and release forms from 131 DZ concordant pairs, by sending letters first to twins who were diagnosed after 1975 and known be alive at last contact. We have also recently sent letters to the next of kin to those who were known to deceased. Currently we have obtained blocks from 36 or 13.7% of these 262 cases.

3) **MZ discordant twins:** We also selected 549 MZ discordant pairs who met the following criteria: a) they were diagnosed after 1975 and we had obtained their pathology report, and b) they had completed the epidemiologic questionnaire that was sent to all females pairs of twins with at least one member with breast cancer who participated in the International Twin Study Registry. We have received blocks from 76 (13.8%) of these cases.

**Correspondence with Hospitals**

Once the signed informed consent and release forms were obtained from a twin, a letter was sent to the hospital along with the release form requesting the tissue blocks, including one that was most representative of the tumor and one that contained normal tissue, such as a lymph node. If the hospital's policies prohibited sending the blocks, we requested that 20 unstained slides be cut from each of the blocks specified, and sent to us. For hospitals not responding follow-up efforts were initiated. Currently we have 71 requests pending with hospitals and have another 88 completed consent forms which we will be sending to the identified hospitals. We are also in the process of returning blocks to the hospitals after we have completed our laboratory studies with them.

Table 1: Status of twin participation and acquisition of blocks/slides by category of pair

| Status | Total | | MZ Con-cordant N | % | DZ Con-cordant N | % | MZ Dis-cordant N | % |
|---|---|---|---|---|---|---|---|---|
| | | | | | Category of Pair | | | |
| Total pairs | 891 | | 211 | | 131 | | 549 | |
| Total individuals (cases) | 1225 | 100.0 | 414 | 100.0 | 262 | 100.0 | 549 | 100.0 |
| | | | | | | | | |
| Resolved cases: | | | | | | | | |
| Blocks/slides rec. | 276 | 22.5 | 164 | 39.6 | 36 | 13.7 | 76 | 13.8 |
| Blocks/slides n/a* | 263 | 21.5 | 142 | 34.3 | 52 | 19.8 | 69 | 12.6 |
| Twin refused | 31 | 2.5 | 17 | 4.1 | 5 | 1.9 | 9 | 1.6 |
| | | | | | | | | |
| Total resolved | 570 | 46.5 | 323 | 78.0 | 93 | 35.5 | 154 | 28.0 |
| | | | | | | | | |
| In process: | | | | | | | | |
| Hosp. Pending | 71 | 5.8 | 18 | 4.4 | 10 | 3.8 | 43 | 7.9 |
| Consent Rec. | 88 | 7.2 | 6 | 1.4 | 11 | 4.2 | 71 | 12.9 |
| Patient Pending | 496 | 40.5 | 67 | 16.2 | 148 | 56.5 | 281 | 51.2 |
| | | | | | | | | |
| Total in process | 655 | 53.5 | 91 | 22.0 | 169 | 64.5 | 395 | 72.0 |

*largely consists of cases who were diagnosed before 1975


**Laboratory Procedures**

Once the blocks (or slides) are received, they are transferred to Dr. Press's Laboratory in padded envelopes which have the Twin ID number, name of submitting hospital, and number of blocks and/or slides provided. This information is logged into a master data file. Variables in this file include information the characteristics of the tissue, number of blocks, number of nodes sampled, and patient information. One H&E slide is cut from each block submitted. Since numerous blocks are sent with some specimens, this enables us to pick a block that is most representative of the tumor and one that is most representative of normal tissue. The 20 unstained slides are then cut from the chosen blocks and are then coated with paraffin so that antigenicity is not lost during storage. After this process has been completed, the blocks are sent back to the hospitals.

Immunohistochemistry: p53 and HER-2/neu

When a specimen is selected to be stained, two slides per analysis are taken. One is for the antibody of interest and the other is used as a negative control. A positive control is used for

every antibody on each day's run. The antibodies are scored on the basis of intensity of staining. HER-2/neu, being a membrane protein, is scored as low (+), over-expressed (++), or highly over-expressed (+++). A tumor was considered to be positive if the staining was either over-expressed or highly overexpressed. P53, ER, and PR, which are nuclear proteins, are scored both by staining intensity and by percentage of cells with that particular intensity, i.e. (27%, +++), (33%, ++), (10%, +). For the initial analyses, we considered a tumor to be positive if 10% or more of the cells were positive.

**Results**

a. Status of block accrual by pair

Among concordant pairs for whom we have received blocks, we have 54 pairs with blocks received from both twins (48 MZ and 6 DZ) and 92 pairs with blocks received from one twin (68 MZ and 24 DZ). Thus, in total we have received blocks from at least one twin for 146 concordant pairs (116 MZ and 30 DZ). In addition we have received blocks from 76 of the 549 discordant MZ pairs.

b. Immunohistochemistry for p53, HER-2/*neu*, ER, and PR

1) Individuals from the concordant pairs

The preliminary results from individuals from the concordant pairs are shown in three ways: 1) percent positive for each biomarker, 2) correlation of the different biomarkers in tumors from the same individual, and 3) concordance of biomarkers within pairs. Of the 200 concordant cases with blocks obtained, immunohistochemistry has been completed on 165 of them. In some cases, the material sent by the hospital was insufficient for analysis while for others, only normal tissue was available. In addition, there are some blocks which have been obtained quite recently for which the immunohistochemistry is not yet complete. (We have also completed immunohistochemistry for 24 of the MZ discordant pairs; however those results are not presented at this time, since we are planning to do so later when a larger sample size will be available).

The percent of tumors that stained positive for each of the biomarkers is shown in Table 2. The overall percent positive ranged from a high of 72.2 for ER and a low of 31.7 for p53. These results are consistent with other reports in the literature. Some important differences by age at diagnosis are evident as ER, PR, and HER-2/neu are both more likely to be positive among those diagnosed at age 50+ vs. those diagnosed <50 years of age; whereas the reverse was true for p53.

Table 2:  Immunohistochemistry Results:  All Individual Cases from Concordant Pairs

| | | Age at Diagnosis | |
|---|---|---|---|
| Percent Positive* for | Total | <50 | 50+ |
| (N) | (165) | (55) | (110) |
| ER | 72.2 | 58.2 | 79.4 |
| PR | 64.6 | 54.6 | 69.7 |
| P53 | 31.7 | 38.9 | 28.2 |
| HER-2/neu | 35.8 | 16.4 | 45.4 |

*Positivity for ER, PR, and p53 defined as expression in 10% or more of cells; positivity for HER-2/neu defined as medium or high expression.

The correlation of positivity for different biomarkers within tumors by age at diagnosis is shown in Table 3A and 3B.  As expected, in both age groups ER and PR were highly correlated ($r=0.63$, $p<.05$ among those diagnosed <50 and $r=0.56$, $p<.05$ among those diagnosed at 50+). P53 was negatively correlated with ER and PR in the younger age at diagnosis group; however in the older group only ER was negatively correlated with p53.  A different association was seen between ER and PR with HER-2/neu by age at diagnosis.  In the younger group these markers were positively correlated with each other, however a slight negative association with ER was seen in the older age at diagnosis group.  In both age groups little correlation was seen between p53 and HER-2/neu.

Table 3A:  Correlation Coefficients Between Tumor Biomarkers: Individual Cases from Concordant Pairs for Age of Diagnosis <50 (N=55).

| | ER | PR | p53 | HER-2/neu |
|---|---|---|---|---|
| ER | 1.00 | | | |
| PR | 0.63* | 1.00 | | |
| p53 | -0.23 | -0.32* | 1.00 | |
| HER-2/neu | 0.28* | 0.21 | -0.05 | 1.00 |

*$p<.05$

Table 3B:  Correlation Coefficients Between Tumor Biomarkers: Individual Cases from Concordant Pairs for Age of Diagnosis 50+ (N=110).

|  | ER | PR | p53 | HER-2/neu |
|---|---|---|---|---|
| ER | 1.00 | | | |
| PR | 0.56* | 1.00 | | |
| p53 | -0.21* | -0.09 | 1.00 | |
| HER-2/neu | -0.18 | 0.006 | 0.09 | 1.00 |

*p<.05

Concordancy of Biomarkers in Within Concordant Pairs

From the 165 twins from the concordant pairs with immunohistochemistry completed, there were 40 pairs (33 MZ and 7 DZ) included with results available from both members of the pair. We next looked to see if these markers were identical between members of the same pair with the supposition being that, if they were, it might indicate that predisposing genetic factors were largely in control of the development of these markers. Table 4 shows relatively low measures of agreement for each of these biomarkers between members from these concordant pairs. Kappa, which takes into account chance agreement, was the highest for ER (0.42, p<.05), and lowest for HER-2/neu (0.19). By age (not shown), Kappa values were higher among the younger age at diagnosis group, especially for PR.

Table 4:  Measures of Agreement Between Biomarkers from Members of Concordant Pairs with Tissue Analyzed from Both Twins

| 40 pairs | Percent Agree | Percent Expected | Kappa |
|---|---|---|---|
| ER | 73.8 | 54.8 | 0.42* |
| PR | 59.0 | 48.7 | 0.20 |
| P53 | 69.2 | 57.3 | 0.28 |
| HER-2/neu | 62.5 | 53.6 | 0.19 |

*p<.05

The relatively low agreement beyond that which would be expected by chance suggests that environmental factors and/or chance may play a role in the development of these tumor characteristics, even in this group of twins from concordant pairs.

b.  Association with Risk Factors

To study the possible role of environmental factors on the development of these biomarkers, we utilized risk factor information that was obtained from the twins when they first entered the Registry.  Using all of the 165 cases from concordant pairs, we developed separate logistic regression models for each biomarker as the dependent variable to calculate adjusted odds rations that measure the association between each biomarker and selected risk factors.  We included parity, nulliparity, age at menarche, OC use, breast feeding, and age at first full term pregnancy in the models.

Table 5 shows the most interesting preliminary finding to emerge from these analyses, which is that breast feeding appears to protect against developing a tumor with a p53 mutation.  The mechanism by which this may occur is unknown.  This supports a previous study which found that prolonged lactation was associated with a 40% reduction in risk of p53+ tumors (van der Kooy, K., Rookus, M., Peterse, H., and Leeuwen, F. p53 protein overexpression in relation to risk factors for breast cancer, Am J. Epidemiol 1996, 144:924-33).

Table 5:  Adjusted Odds Ratios Measuring the Association of Breast Feeding for Each Tumor Biomarker:  Cases from Concordant Pairs by Age at Diagnosis.

| Breast Fed | Age at Dx <50 | | Age at Dx 50+ | |
| 1+ infant(s) | Adjusted OR(1) | Adjusted OR (2) | Adjusted OR (1) | Adjusted OR (2) |
|---|---|---|---|---|
| ER | 0.95 | 1.59 | 1.25 | 1.87 |
| PR | 1.64 | 3.46 | 1.39 | 1.64 |
| P53 | 0.12* | 0.10* | 0.25* | 0.18* |
| HER-2/*neu* | 1.20 | 1.67 | 1.24 | 1.06 |

*p>.05
(1) All cases: adjusted for parity, nulliparity, age at menarche, and OC use.
(2) Parous cased only: adjusted for above, plus age at first full term pregnancy.

Additional analyses of this finding and of the association of other risk factors with the tumor biomarkers are ongoing.

## C. CONCLUSIONS

We have continued to obtain tissue blocks from the 207 MZ and 131 DZ concordant twin pairs and the 549 MZ discordant pairs. Once all efforts have been expended to locate the next of kin without success, we will contact the hospitals directly.  The laboratory procedures for processing the blocks and the immunohistochemistry procedures will continue.  We are now beginning the

process of identifying the specific p53 mutations in these tumors. Due to improving technology, we now plan to sequence the entire gene of all cases whether of not they stained positive for p53 (and not rely on SSCP to identify the exon to sequence). We will also implement the procedures to do FISH analyses to quantify HER-2/*neu* gene amplification. Epidemiologic analyses of the concordant pairs and discordant pairs (stratified by tumor biomarker results) will be completed.

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